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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Breast cancer is the most common life-threatening cancer and second leading cause of cancer deaths in Western women. Death usually results from the complications caused by secondary tumors that result from termed invasion and metastasis, processes that depend on the secretion of proteinases. The level of one such proteinase inhibitor, named TIMP-1, predicted to inhibit invasion, paradoxically has been found to be higher in breast cancers of patients that more often relapse and die as compared to patients that survive. This suggests that this protein may have multiple functions that include both inhibition of cancer promoting proteinases and stimulation of cell-signaling pathways that promote cancer progression.

In support of this hypothesis, we have determined that overexpression of TIMP-1 activates Ras, the MAPK pathway and induces epithelial-mesenchymal transition (EMT), a process important in invasion of tumor cells into surrounding tissues. Furthermore, TIMP-1 expressing cells were more motile and overexpressed a protein named PAI-1 that has been implicated in breast cancer progression. These studies support clinical studies demonstrating that high tissue levels of TIMP-1 correlate with a bad prognosis and suggest that the reason for this may be because TIMP-1 activates signaling pathways that promote EMT. Future studies will examine this hypothesis.

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INTRODUCTION

The goal of our research is determine how TIMP-1 contributes to breast cancer progression. This MMP inhibitor has historically been considered an anti-cancer protein, but a number of published studies now demonstrate this protein can also act to promote cancer progression. We hypothesize that TIMP-1 can stimulate invasion of breast tumor cells via activation of Ras and other downstream pathways. We further propose that a new version of TIMP-1 can be genetically engineered that will still inhibit MMP function but will be inactivate in promoting cancer progression. The following represents a one year progress report of our studies.

BODY (Review of data obtained July 1, 2003 to June 30, 2004)

Overview of this Section:

We have generated interesting data applicable to Task 1 and Task 2, as summarized in the following sections. In the first section, we review data we obtained that suggests that TIMP-1 overexpression can cause epithelial mesenchymal transition (EMT), a key change leading to invasion and metastasis. This is very novel and exciting data, and we will be submitting a manuscript soon that describes these results. It is already known that high levels of TIMP-1 correlate with bad prognosis for breast cancer patients, and this new data suggests a possible reason why this is true. Figures in support of the text can be found at the end of the BODY section.

I. Publishable Data

TIMP-1 overexpression in MCF10A cells induces Epithelial to Mesenchymal Transition (Relevant to Task 1a, 1b). TIMP-1 is overexpressed in breast cancer specimens and is correlated with poor prognosis, suggesting that TIMP-1 may have an adverse effect during cancer progression (1-4). The majority of the literature focuses on TIMP-1's inhibitory effects towards MMPs, whereas little is understood about TIMP-1's potential involvement in the development of a malignant phenotype. In order to gain a better understanding of TIMP-1 function and its oncogenic properties, we examined MCF10A cells, which are a normal, non-tumorigenic cell line, that were created to overexpress TIMP-1 (5,6). Light microscopy revealed that morphological differences were highly pronounced. MCF10A Neo (control cells) and TIMP-1 overexpressing clones (#3 and #29) were fixed and stained for actin. Vector control cells displayed the typical cobblestone (Figure 1 a, c) morphology, whereas the stable MCF10A clone #3 overexpressing TIMP-1 appeared to have a mesenchymal phenotype (Figure 1 b, d). This was evidenced by long membrane protrusions and increased actin stress fibers formation. Clone #29 also demonstrated an identical morphological and phenotype (not shown). Additionally, the TIMP-1 expressing cells did not appear to make stable contacts with neighboring cells.

Immunofluorescence microscopy was employed to determine if the TIMP-1 overexpressors contained less E-cadherin and/or β-catenin, thus accounting for the lack of association with each other. MCF10A Neo expressed E-cadherin and -catenin predominantly at cell-cell junctions (Figure 1 e and g). Interestingly, E-cadherin and -catenin were barely detectable in the TIMP-1 overexpressing clones (Figure 1 f and h). These observations were confirmed by western blot analysis of MCF10A Neo and TIMP-

1 clone #3 and #29 (Figure 2), indicating the complete loss of E-cadherin and a significant decrease in the levels of β-catenin in the TIMP-1 expressing clones.

Loss of E-cadherin and -catenin are associated with a mesenchymal phenotype (7). In order to determine if TIMP-1 is inducing EMT in MCF10A cells, antibodies were used against, vimentin, a marker associated with a mesenchymal phenotype in western blot analysis. Vector control cells had no expression of vimentin, whereas the TIMP-1 clones expressed high levels of vimentin. Furthermore, whereas control cells expressed high levels of cytokeratin, the TIMP-1 overexpressors displayed a complete loss of expression of the epithelial marker, cytokeratin (Figure 2).

TIMP-1 overexpression increases motility of MCF10A cells (Relevant to Task 1a and 1b). EMT is associated with increased focal adhesions and motility (7). Immunofluorescence microscopy revealed increased levels of phospho-paxillin (Figure 3 a, b and c) and phospho-tyrosine (Figure 3 d, e and f) expression in the two TIMP-1 expressing clones. Phospho-paxillin was undetectable in MCF10A Neo (a), whereas it was predominantly expressed at the ends of actin stress fibers in TIMP-1 #3 clone (b, c) (arrows). Phospho-tyrosine was localized justanuclear in MCF10A Neo (d), while in TIMP-1 clone #3, phospho-tyrosine was expressed not only throughout the cells, but also at the ends of actin stress fibers (e, f) (arrows).

In order to assess the effect of TIMP-1 on motility, $2x10^4$ vector control and TIMP-1 #3 and #29 cells were plated on top of inserts in a 24-well Transwell plate. The cells were incubated for 12 hours and the cells were fixed with 4% paraformaldehyde. The cells remaining on the top of the insert were removed with a cotton swab, and the remaining cells on the bottom of the insert were stained with crystal violet. Cells were counted in 5 random fields and totaled. Figure 4 reveals that TIMP-1 clones #3 and #29 had a significantly higher number of cells which migrated to the undersurface of the insert when compared to vector control.

TIMP-1 induces a redistribution of lysosomes (Relevant to task 1a and 1b). The cellular location and secretion of degradative enzymes is important during the invasive and migratory process of tumor cells. Lysosomes are acidic vesicles which have been suggested to actively secrete proteinases into the extracellular space during migration and invasion (8). Immunofluorescence microscopy revealed that MCF10A TIMP-1 clones #3 and #29 displayed a redistribution of LAMP-1 positive lysosomes when compared to vector control cells. Lysosomes in MCF10A Neo were juxtanuclear (Figure 5 a, c), whereas in the TIMP-1 clones, the lysosomes were distributed throughout the cell (Figure 5 b, d), especially at the cellular periphery, and at the ends of membrane extensions (arrows). Immunofluorescence microscopy also revealed that late and early endosomes identified using the markers MPR and EEA1, respectively, localized to juxtanuclear positions in control and TIMP-1 expressing cells, suggesting that only lysosomes are induced to redistribute.

II. Additional Preliminary Data

Overexpression of TIMP-1 activates the Ras and the MAPK pathways and increases the expression of gelatinases and plasminogen activator inhibitor-1 (PAI-1) (Relevant to Task 2a). We have confirmed the published data of Dr. Kim (Wayne State

University) demonstrating that TIMP-1 expressing cells contained higher levels of Ras-GTP and Erk-P (results not shown). Interestingly, these cells also demonstrate increase secretion of MMP-2 and MMP-9 (results not shown). Finally, to begin to analyze global gene expression changes in cells expressing TIMP-1, we have utilized the superarray filters. We noted a number of changes both up and down in expression of genes responsive to increased TIMP-1 levels. Notably, PAI-1 levels were increased at least 5 fold at a protein and RNA level in cells overexpressing TIMP-1 (results not shown). This is very interesting, since PAI-1 has been implicated in the progression of breast cancer and little is known about the factors that regulate its expression in malignant cells. We are currently analyzing cells that overexpress PAI-1 to determine if EMT occurs and if PAI-1 is a downstream mediator of TIMP-1 action.

Generation of Cell-Lines Inducibly Overexpressing TIMP-1 (Relevant to Task 1a). We have had trouble obtaining the MCF-7 cell-lines that stably overexpress TIMP-1 and our MCF-10A overexpressors have become unstable. Therefore, we are implementing the Tet-off inducible system to conditionally overexpress TIMP-1. We have already cloned the DNA fragment encoding TIMP-1 into the Tet responsive promoter and we have ordered the MCF-7 and MCF-10A cell-lines that stably express the Tet protein. We will select for clones that contain the TIMP-1 plasmid that demonstrated controlled but robust expression of TIMP-1 following removal of tetracycline from the media. Suitable MCF-7 clones conditionally expressing TIMP-1 will be used in animal studies beginning in year two.

KEY RESEARCH ACCOMPLISHMENTS:

1. Obtained two clones of MCF10A cells which overexpress TIMP-1.

2. Determined that TIMP-1 overexpression in MCF10A cells results in a mesenchymal phenotype compared to vector controls through immunofluorescence staining.

3. The TIMP-1 clones had decreased levels of E-cadherin and -catenin as evidenced by immunofluorescence microscopy and western blot analysis.

4. TIMP-1 overexpression in MCF10A cells resulted in increased vimentin levels and decreased cytokeratin expression compared to vector control, both hallmarks of cells which have undergone epithelial to mesenchymal transition.

5. The MCF10A TIMP-1 clones had high levels of phospho-tyrosine and phospho-paxillin as noted by immunofluorescence.

6. TIMP-1 overexpression in MCF10A cells increased their motility compared to vector control in the modified Boyden Chamber migration assay.

7. Overexpression of TIMP-1 in MCF10A cells resulted in a redistribution of lysosomes from justa-nuclear to the cellular periphery, especially at sites of membrane protrusions. No changes in localization were observed for early, recycling or late endosomes.

8. Array analysis using SuperArray Human Tumor Metastasis arrays revealed that PAI-1 is overexpressed in the TIMP-1 clones. This was confirmed by RT-PCR analysis.

9. TIMP-1 overexpression activates the ras/ERK activity in MCF10A cells.

10. Began creating doxycycline-inducible MCF10A and MCF-7 cell lines. Created vectors for inducible TIMP-1 expression.

REPORTABLE OUTCOMES:

RLH Bigelow, JA Cardelli. 2004. TIMP-1 Induces Epithelial to Mesenchymal Transition in MCF10A Breast Epithelial Cells. Abstract for the *"95th meeting for the American Association for Cancer Research"*. Orlando, FL.

RLH Bigelow, JA Cardelli. TIMP-1 Induces Epithelial to Mesenchymal Transition in MCF10A Breast Epithelial Cells. Manuscript in preparation.

CONCLUSIONS:

TIMP-1 is typically believed to function as an inhibitor of the malignant phenotype through its ability to inhibit matrix metalloproteinase activity. The published observations that TIMP-1 is overexpressed in many different types of cancer and is associated with poor prognosis, suggests that TIMP-1 also possesses a pro-malignant activity. EMT (Epithelial to Mesenchymal Transition) is believed to be the initiating stage leading to tumor metastasis. Gaining a better understanding of the processes leading to EMT will allow us to better treat metastatic disease. We have demonstrated that TIMP-1 induces epithelial to mesenchymal transition (EMT) in MCF10A breast epithelial cells as noted by decreased E-cadherin, -catenin and cytokeratin levels and increased vimentin expression. TIMP-1 also increased the motility of MCF10A cells.

We observed a redistribution of lysosomes to the periphery of the TIMP-1 overexpressing clones, suggesting that this phenomenon may play a crucial role in the release of ECM remodeling enzymes and the invasive process. Interestingly, we also observed lysosome redistribution in cells exposed to HGF, which triggers EMT in DU145 prostate tumor cells. This suggests that lysosome redistribution may be functionally connected to EMT triggered by a variety of agents. Future studies are aimed at assessing the importance of lysosomal redistribution in the invasive process and the signaling pathways responsible through a recently funded grant.

The mechanisms by which TIMP-1 is able to induce EMT are currently being investigated. Preliminary observations suggest that TIMP-1 can activate the ras/ERK pathway in MCF10A cells. In addition, PAI-1 was overexpressed in the TIMP-1 clones when compared to vector control. Future studies are aimed at assessing the importance of the ras/ERK pathway and PAI-1 in TIMP-1 function and induction of EMT. Doxycycline inducible MCF10A and MCF-7 cells will be used to overexpress TIMP-1 in order to determine the temporal effects of TIMP-1.

Our data revealing that TIMP-1 can induce EMT provides a possible explanation for the clinical data showing high levels of TIMP-1 associated with poor prognosis. Synthetic inhibitors resembling TIMP-1 have been used in the clinic albeit with poor results. Our accomplishments and future studies will provide information concerning the function and the signaling pathways activated by TIMP-1. These studies aim to create better alternative strategies to target TIMP-1's pro-malignant properties and metastatic disease.

FIGURES

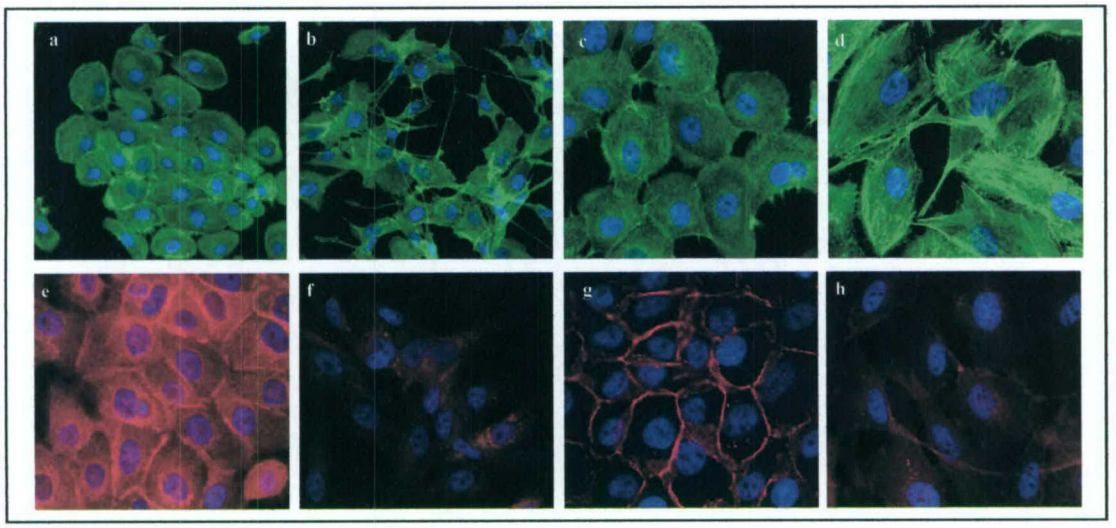

Figure 1: TIMP-1 overexpression induces a mesenchymal phenotype and results in loss of E-cadherin and β-catenin expression. Immunofluorescence of MCF10A neo (a, c, e, g) and MCF10A TIMP clone #3 cells (b, d, f, h). Actin staining (green) at 20x (a, b) and 40x (c, d) reveals a mesenchymal appearance in the TIMP-1 overexpressing clones as noted by increased actin stress fiber formation and a hummingbird phenotype. E-cadherin (e, f- red) and β-catenin (g, h- red), at 40x, are present at cell-cell junctions in MCF10A Neo (e, g) and are lost in the TIMP-1 clone #3 (f, h). Cells were counterstained with DAPI in all images (blue). Bar = 15 μM in a, b and 7.5 μM in c-h.

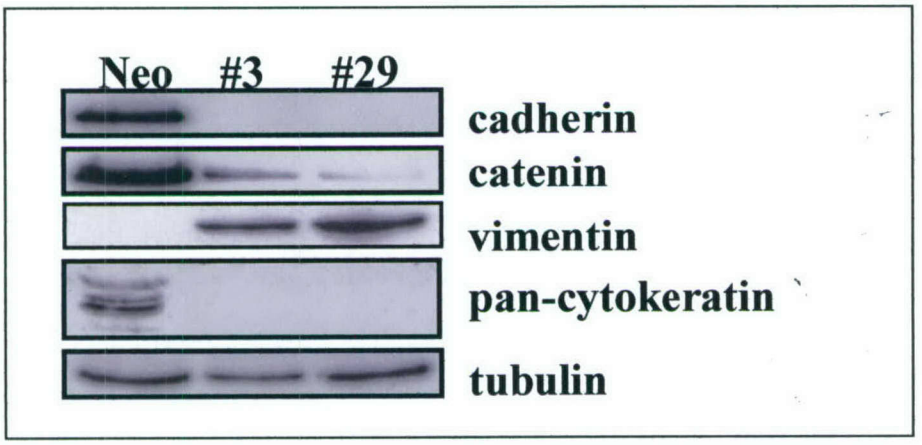

Figure 2: TIMP-1 overexpression induces EMT in MCF10A cells. Protein lysates (10 μl) of MCF10A Neo, TIMP-1 #3 and TIMP-1 #29 were run on an SDS-PAGE gel, transferred to nitrocellulose and blotted with antibodies corresponding to the indicated proteins. TIMP-1 overexpression reduced E-cadherin and β-catenin expression. The mesenchymal marker, vimentin, was increased in the TIMP-1 overexpressing clones, whereas an antibody to pan-cytokeratin reveals a decrease in the TIMP-1 clones. Tubulin was used as a load control.

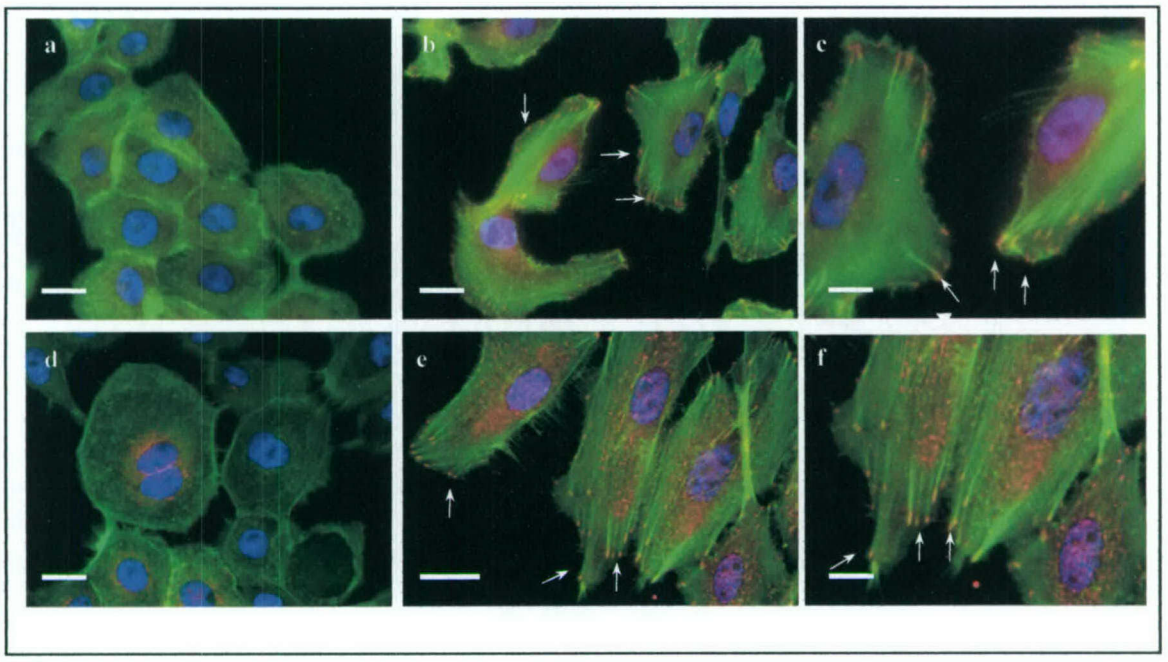

Figure 3: MCF10A TIMP clones #3 and #29 have higher levels of phospho-paxillin and phospho-tyrosine. Immunofluorescence of MCF10A neo (a, d) and MCF10A TIMP-1 #3 (b, e) with antibodies to the phosphorylated forms of paxillin (a, b) at 40x and phospho-tyrosine (d, e) at 40x, both in red, reveals that TIMP-1 induces increased phospho-paxillin and phospho-tyrosine at the ends of actin stress fibers (green) (arrows) compared to vector control cells. (c) phospho-paxillin at 60x for MCF10A TIMP-1#3 (f) phospho-tyrosine at 60x for MCF10A TIMP-1#3. Nuclei were stained with DAPI (blue). Bar = 7.5 μM in a-b and d-e. Bar = 5 μM in c and f.

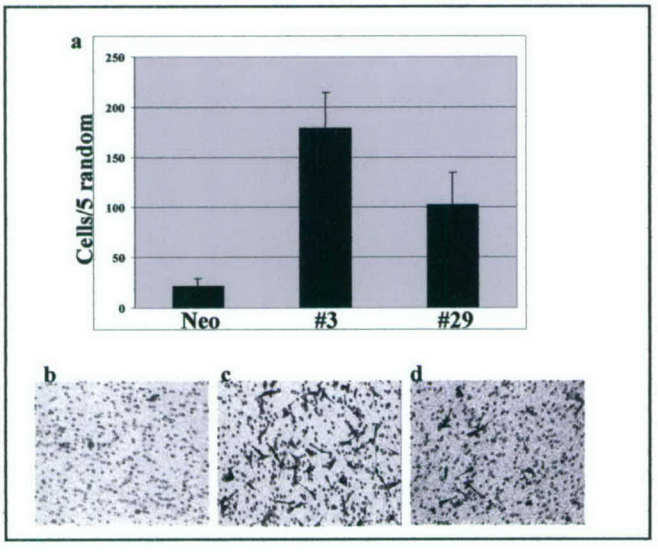

Figure 4: MCF10A TIMP#3 and #29 are more motile than vector control. $2x10^4$ cells were plated on top of Costar Transwell Inserts with serum containing media on the top of the insert and in the bottom of the well. The inserts were incubated for 12 hours at

37°C and the cells were fixed with 4% PFA and stained with crystal violet. The cells remaining on the top of the insert were removed with a cotton swab. The cells which migrated to the bottom of the insert were counted in 5 random fields per insert and totaled. The experiment was performed in triplicate and averages were taken and plotted (a). TIMP-1 overexpression stimulated cell migration to the bottom of the insert in clones #3 (c) and #29 (d) compared to vector control (b).

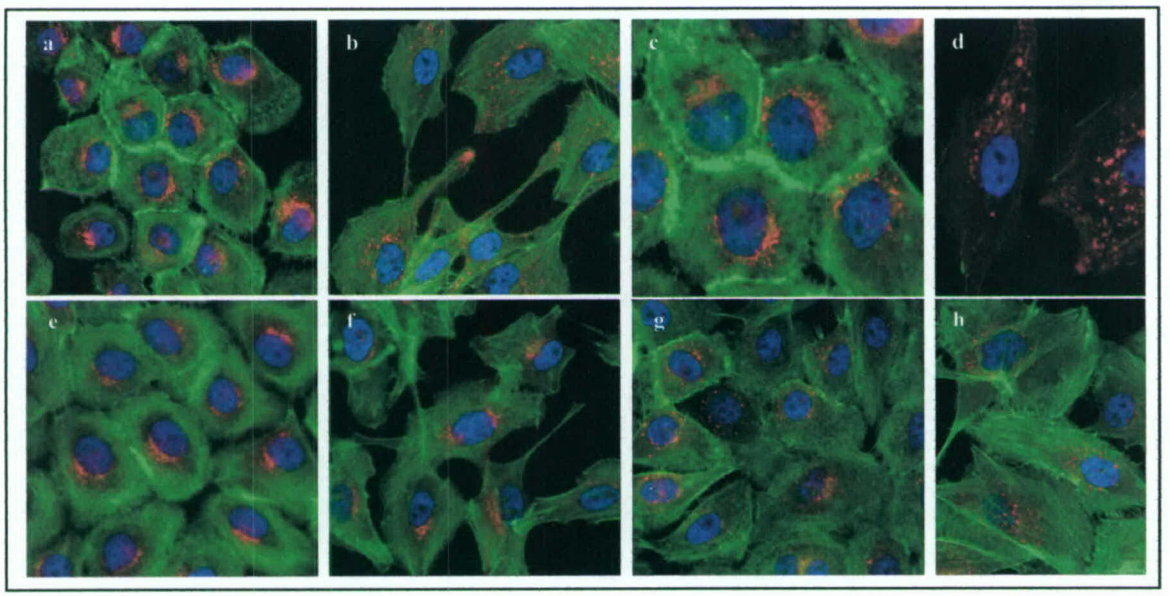

Figure 5: TIMP-1 overexpression results in a redistribution of lysosomes. Immunofluorescence of MCF10A neo (a, c, e, g), MCF10A TIMP-1 #29 (b, d) and TIMP-1 #3 (f, h). Staining with LAMP-1 (red) at 40x (a, b) and 60x (c, d) reveals that lysosomes are justanuclear in MCF10A Neo (a, c). TIMP-1 overexpession results in a redistribution of LAMP-1 positive lysosomes throughout the cell and to the cellular periphery (arrows). Please note LAMP-1 positive vesicles at the tips of the filapodia in figure (b) (arrow). Actin intensity was decreased in (d) to observe redistribution of LAMP-1. Immunofluorescence of MPR (e, f) and EEA1 (g, h), at 40x, do not reveal a redistribution of late or early endosomes, respectively. Cells were counterstained with phalloidin (green) and nuclei were stained with DAPI (blue) in (a) through (h). Bar = 7.5 μM in a-b and e-h. Bar = 5 μM in c-d.

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